Dominant Parasympathetic Modulation of Heart Rate and Heart Rate Variability in a Wild-Caught Seabird

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ABSTRACT

Heart rate (HR) and heart rate variability (HRV) provide noninvasive measures of the relative activity of the parasympathetic nervous system (PNS), which promotes self-maintenance and restoration, and the sympathetic nervous system (SNS), which prepares an animal for danger. The PNS decreases HR, whereas the SNS increases HR. The PNS and SNS also contribute to oscillations in heartbeat intervals at different frequencies, producing HRV. HRV promotes resilience and adjustment capacity in the organism to intrinsic and extrinsic changes. Measuring HRV can reveal the condition and emotional state of animals, including aspects of their stress physiology. Until now, the functioning of the PNS and SNS and their relationship with other physiological systems have been studied almost exclusively in humans. In this study, we tested their influence on HR and HRV for the first time in a wild-caught seabird, the streaked shearwater (*Calonectris leucomelas*). We analyzed electrocardiograms collected from birds carrying externally attached HR loggers and that received injections that pharmacologically blocked the PNS, the SNS, or both, as well as those that received a saline (sham) injection or no injection (control). The PNS strongly dominated modulation of HR and also HRV across all frequencies, whereas the SNS contributed only slightly to low-frequency oscillations. The saline injection itself acted as a stressor, causing a dramatic drop in PNS activity in HRV and an increase in HR, though PNS activity continued to dominate even during acute stress. Dominant PNS activity is expected for long-lived species, which should employ physiological strategies that minimize somatic deterioration coming from stress.

Keywords: autonomic nervous system, heart rate, heart rate variability, stress, shearwater.

Introduction

Animals have evolved complex physiological mechanisms to optimally allocate limited resources to survival and reproduction while coping with a constantly changing environment. In vertebrates, the autonomic nervous system (ANS) plays a key role in this process, coordinating physiological responses to external and internal changes while maintaining a dynamic equilibrium and regulating energy expenditure (Porges 1992; Kuenzel 2015). The ANS is composed of two neural branches, the parasympathetic nervous system (PNS) and the sympathetic nervous system (SNS). The PNS promotes self-maintenance by promoting restoration and energy conservation, while the SNS prepares the individual for external challenges by mobilizing body reserves and activating muscular action (see physiological effects of ANS-mediated stress response in fig. A1; summarized from Porges 1992; Wingfield 2013). The PNS and SNS connect the brainstem to a variety of organs throughout the body, notably, the heart (Taylor et al. 2014; Kuenzel 2015).

Though the pulse of the heartbeat is initiated by the firing of the sinoatrial node in the heart, which produces an intrinsic heart rate (HR) of very regularly spaced heartbeat intervals at a moderate pace, the heart rhythm is further modulated by the efferent neurons from the PNS and the SNS (Shaffer et al. 2014; Taylor et al. 2014). In the heart, the PNS reduces HR and the SNS increases HR (Brindle et al. 2014; Kuenzel 2015). The PNS and, to a lesser extent, the SNS also generate oscillations in heartbeat intervals at different frequencies and amplitudes, thus producing heart rate variability (HRV). This complex and changing heart rhythm promotes resilience and adjustment capacity in the organism to intrinsic and extrinsic changes (McCraty and Childre 2010). Indeed, reduced HRV reflects a reduced ability to appropriately respond to challenges such as exercise or stressors (reviewed in Shaffer et al. 2014). Thus, detailed measurements of cardiac function can potentially reveal the condition and emotional state of animals, including their stress physiology (Porges 1992; Koolhaas et al. 1999; Cyr et al. 2009).

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Until now the functioning of the ANS and its relationship with other physiological systems, behavior, and/or the stress response have been studied almost exclusively in humans (reviewed in Shaffer et al. 2014) and in a few domesticated animals (reviewed in von Borell et al. 2007). Despite the relevance of HR and HRV measures to wide-ranging physiological and behavioral processes, a large gap exists for similar work on wild animals (but see Cyr et al. 2009; Dickens and Romero 2009; Taylor et al. 2014). This is mainly due to difficulties in obtaining the high-resolution electrocardiogram (ECG) data required for analyzing ANS activity. However, recent technological advances have permitted the development of animal-borne ECG loggers that can be externally attached to free-living animals and can record ECG under both natural and captive conditions (Muramoto et al. 2004; Campbell et al. 2006; Ropert-Coudert et al. 2006, 2009; Yamamoto et al. 2009).

Here, we investigated the ANS regulation of HR and HRV in wild-caught streaked shearwaters (*Calonectris leucomelas*). Shearwaters are long-lived pelagic seabirds that have become a popular model for behavioral and physiological field research (e.g., Dell'Ariccia et al. 2014; Ogawa et al. 2015). They breed in large, easily accessible colonies, are easy to capture and observe, and have relatively large body mass that enables the attachment of biologging instruments for recording their movements and physiological parameters (Müller et al. 2014, 2015; Yoda et al. 2014). The objectives of our study were to determine the activity and relative importance of the PNS and SNS in regulating cardiac function in adult streaked shearwaters. To this aim, we calculated frequency and time domain indexes of HR and HRV during resting conditions, following the short-term acute-stress situation provoked by a saline injection and after pharmacologically blocking the actions of either one branch or two of the branches of the ANS.

Until now, few avian studies have examined HRV by comparing isolated PNS or SNS activity with that of total ANS blockade; most studies compared it to the net autonomic balance that occurs after a saline treatment (in which both the PNS and the SNS are active; Cyr et al. 2009; Yamamoto et al. 2009; reviewed in Kuenzel 2015). As PNS and SNS effects are often antagonistic (Brindle et al. 2014), net autonomic balance of HRV under saline injection is often not simply the sum of isolated PNS activity and isolated SNS activity. Therefore, comparisons of single-branch blockade with a saline sham can make it difficult to accurately quantify isolated PNS or SNS drive. The comparison of single-branch blockade with a saline treatment does, however, reveal whether the PNS or SNS reciprocally suppresses each other's influence on HRV. To the best of our knowledge, this is one of the first avian studies to quantify not only reciprocal suppression of ANS branches but also isolated PNS and SNS drive.

Methods

Birds

Adult streaked shearwaters were captured from a large colony on Awashima Island (38°18′N, 139°13′E), where about 84,000 birds breed (M. Yamamoto, unpublished data), at the end of the breeding season in 2008 and 2010. The sample size was as follows: 4 individuals for a preliminary dose determination study (see appendix), 10 individuals for the main HR and HRV determination experiment, and 5 individuals for respiration rate determination.

For the preliminary dose determination study and the HR and HRV determination experiment, birds were transported to a field station located on the island and kept in individual cages in an experiment room for a maximum period of 5 d. Body mass was monitored every day during captivity with a spring balance to the nearest 5 g. After the experiments, birds were force-fed 45 g of fish (Japanese red sea bream, *Pagrus major*) and kept under observation for 24 h before being released at the breeding colony.

HR and HRV Determination Experiment

ECG Data Loggers. For HR and HRV determination, shearwaters were equipped with miniaturized externally attached ECG data loggers (UWE-200ECG, 12-bit resolution, 105 mm × 20 mm, 52 g, Little Leonardo). These devices can record ECGs continuously for up to 4.5 h at a 1-ms sampling interval and can be used to detect heartbeats in birds as shown in Adélie penguins (*Pygoscelis adeliae*; Kuroki et al. 1999), Cape gannets (*Morus capensis*; Ropert-Coudert et al. 2006), and great cormorants (*Phalacrocorax carbo*; Yamamoto et al. 2009). The ECG logger has an 8-MB flash memory and three cables (1-mm diameter) with safety pins soldered to the ends that function as electrodes. The logger records the electric potential difference between two of the electrodes at a range of −5.9 to +5.9 mV with 2.88×10^{-3} mV resolution, and the third electrode is a reference electrode that reduces electric noise. The first two electrodes were pinned to approximately 1 cm of skin above and below the sternum, while the reference electrode was pinned to the skin on the flank. The body of the logger was fixed with plastic ties to feathers on the lower part of the tail. Before beginning the experiments, we checked that the position of the electrodes delivered a correct ECG (presence of the QRS complex) by visually comparing the output of the logger with the signal recorded by an analogue ECG monitor (Parama-Tech).

ANS Blockade Procedure. As ANS activity and thus HR can be affected by thermoregulation (Stauss 2003; von Borell et al. 2007), HR and HRV determination was performed at a room temperature of 25°–26°C, known to fall within the thermoneutral zone of temperate procellariiform species (Ochoa-Acuña and Montevecchi 2002). In order to eliminate the effect of a postfeeding increase in internal temperature, birds were kept in captivity and fasted for 24 h before the experiment. Thereafter, ECG loggers were attached and birds were placed in individual closed boxes in a silent room to recover for two more hours. Then the cables of the electrodes were connected to the ECG data logger and ECG recordings were initiated before the remaining experimental procedures, as follows.

A first group of five birds (group 1) received three consecutive treatments separated by a 24-h interval, by injection into their tarsal vein, following a design based on Yamamoto et al. (2009). Namely, birds were injected with (1) a saline solution (0.9%) to

determine the effect of injection (sham treatment); (2) atropine (0.3 mg kg^{-1}), an acetylcholine-receptor antagonist, which inhibits the PNS; and (3) propranolol (0.2 mg kg^{-1}), a β-blocker, which inhibits the SNS. ECG recordings began 1–3-min before injection (handling during injection lasted 3–5 min) and continued for 50 min postinjection. Birds were kept in dark boxes in a quiet room between daily treatments.

A second group of five more birds (group 2) received no initial injection (i.e., resting controls), with ECGs being recorded 1 h to obtain the basal signal while birds were resting in individual dark boxes. Thereafter, these birds received a simultaneous injection of both atropine (0.3 mg kg^{-1}) and propranolol (0.2 mg kg^{-1}) into the tarsal vein to inhibit PNS and SNS activity (i.e., total ANS blockade). After the injection, the ECG loggers continued to record for an additional 50 min.

Analysis of the ECG Data. ECG data were analyzed using Igor Pro, version 6.03 (Wavemetrics). Von Borell et al. (2007) suggested analyzing at least 5 min of continuous HR recordings for a reliable assessment of HRV. Here, we analyzed 9-min intervals, since this was the maximum interval of continuous ECG recording without noise, with R peaks clearly identifiable. R peaks associated with individual heartbeats were detected using a purpose-written macro on Igor Pro (Sakamoto et al. 2009), edited manually, and then used to compute interbeat intervals (IBIs).

To identify the phase during which the treatments had maximum effect, three different time intervals of 9 min of continuous ECG recordings were analyzed within the following time windows: phase 1 (within 4–15 min after injection), phase 2 (within 16–31 min after injection), and phase 3 (within 35–49 min after injection; see appendix). Phase 1 was selected for HRV index calculations, based on the observed effects of atropine and propranolol on HR (see appendix) and in accordance with previous studies on HRV in birds (Matsui and Sugano 1987; Cyr et al. 2009). Phases 2 and 3 were not used in further analyses. For the resting control group, a 9-min interval starting at least 30 min after logger attachment was used for HRV index calculation. All groups contained $n = 5$, except for the saline (sham) treatment, which contained $n = 4$ because the logger became partially detached during ECG recording in one bird.

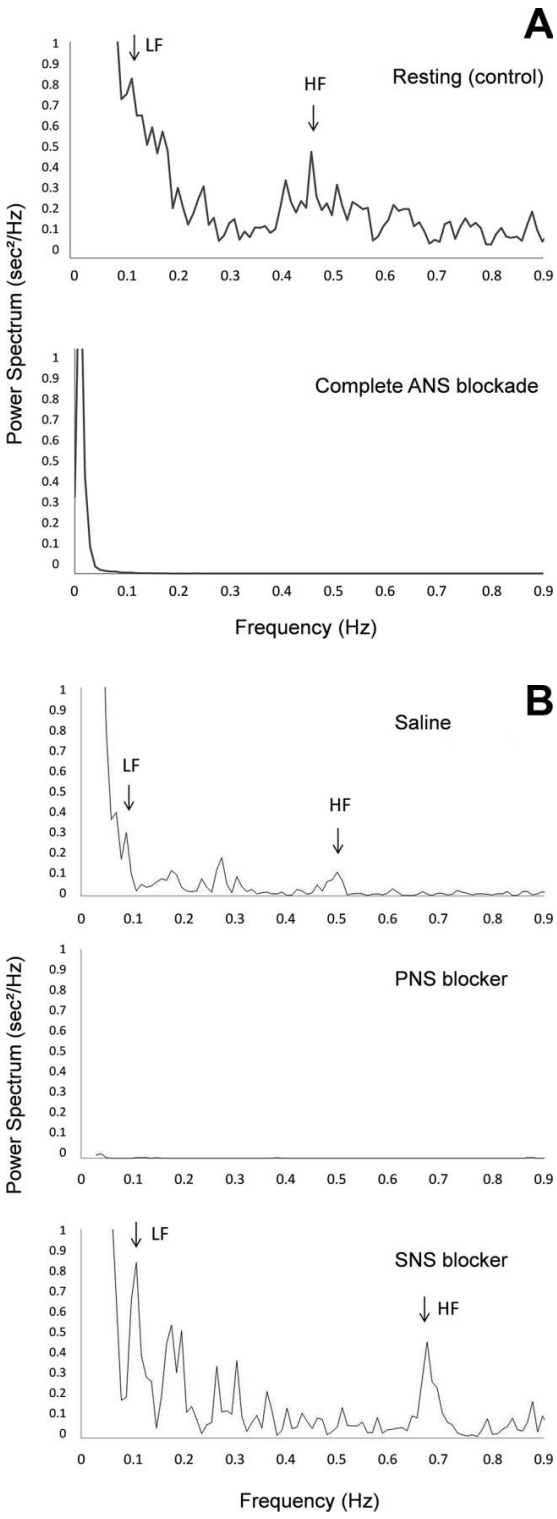
HR and Coefficient of Variation of HR. HR (beats per minute [bpm]) of streaked shearwaters was calculated as 60 divided by the number of IBIs (s). The coefficient of variation (CV) represents the overall variability of the series of IBIs and reflects combined contributions of PNS and SNS activity to HRV. CV was calculated as the standard deviation (SD) of HR divided by the mean HR. HR is expected to be at maximum under sole SNS control, when PNS activity is inhibited, and is expected to be at minimum under sole PNS control, when SNS activity is inhibited. PNS and SNS drive (i.e., the isolated contributions of the PNS or SNS) can be calculated from the mean HR after SNS or PNS blockade, respectively, as the percentage difference from intrinsic HR (the HR during total ANS blockade after injection with both propranolol and atropine; Altimiras et al. 1997; Yamamoto et al. 2009).

Frequency Domain Analysis of HRV. The 9-min sampling intervals of IBIs were interpolated using a cubic spline to produce a tachogram, which was then resampled at 100 ms so that consecutive data points were evenly spaced over time. A power spectral density estimation of HRV was performed involving a fast Fourier transform of the resampled data over periods of 1,024 data points (102.4 s) with a step of 5.12 s. The power spectrum was used to reveal oscillations occurring in the IBIs over time at different frequencies: when the power spectrum is plotted against frequency, each peak corresponds to the frequency at which an oscillation occurs, and the height of the peak (power) corresponds to the square of the amplitude of the oscillation. Therefore, the most important, clearly definable peaks are the most pronounced oscillations in the ECG wave. The area under the entire curve (total power) represents all the variability in IBIs with peaks occurring at the frequencies of relatively important (high-amplitude) oscillations.

Two spectral peaks are relevant for these analyses: those peaks that reflect actions of PNS and/or SNS. The peaks occur at different frequencies due to functional disparities between the two ANS branches. The higher-frequency (HF) peak reflects periodicities in HRV corresponding to respiration rate (respiratory sinus arrhythmia) and is modulated by rapid signals from the PNS (reviewed by Stauss 2003): during exhalation, the HR slows down, and this pattern of HRV has been suggested to increase the effectiveness of respiratory gas exchange (Stauss 2003; Taylor et al. 2014).

In the SNS, however, norepinephrine has a relatively slow exocytotic release from the sympathetic nerves through which SNS regulates cardiac activity, and its transduction also involves a secondary messenger, adenylyl cyclase, which further slows the process (reviewed by von Borell et al. 2007). Therefore, the SNS influences only the lower-frequency (LF) domain, though the PNS contributes to the LF component as well (Malik et al. 1996; von Borell 2007). The LF : HF ratio therefore can reflect SNS-PNS balance (Pagani 1984, 1986; von Borell et al. 2007; Ropert-Coudert et al. 2009).

Determination of Frequency Domain Indexes and Resting Respiration Rate. For calculations of frequency domain indexes, the delimitation between the LF and HF domains was set according to the occurrence of peaks in the power spectrum (see "Results"; fig. 1; table 1) and to previous results on HRV investigations in bird (e.g., Yamamoto et al. 2009; Shah et al. 2010). Furthermore, in order to identify respiratory sinus arrhythmia in the HRV signal, five adult streaked shearwaters were captured and put in individual cages close to the breeding colony in October 2010. Chest movements were filmed with a video camera (Lumix DMC-FX33, Panasonic) through a small opening on the top of the cage slightly illuminated by a LED light, while the birds were resting. The birds were first allowed to acclimate to the cage for 30 min, filmed for another 30 min, and then were released at the colony. Respiration rate was obtained by counting chest movements during playback of the tape while recording time with a stopwatch. Measures were taken on a per-minute basis for a time interval of 10 min.



Time Domain Analysis of HRV. Time domain indexes were calculated directly from the IBI data and included the SD of all IBIs (SDNN), which reflects activity of both PNS and SNS; the SD of the differences between successive IBIs (RMSSD), which reflects PNS activity; and the SDNN to RMSSD ratio, which indicates the balance between the two branches of the ANS (table 1; Malik et al. 1996; von Borell et al. 2007; Ropert-Coudert et al. 2009; Kjaer and Jørgensen 2011; Shaffer et al. 2014). These indexes were calculated using Ethographer v.1.3 (Sakamoto et al. 2009).

Statistical Analysis

Analyses were conducted using R 2.15.1 (R Core Team 2012) using the "stats" package. Distributions of HR and HRV indexes were tested for normality by Shapiro-Wilk tests, and, depending on the results, parametric or nonparametric tests were used for comparison between groups. Namely, (i) paired Wilcoxon signed-rank tests were used to compare indexes within group 1 birds (saline, atropine, and propranolol treatments), (ii) paired *t*-tests were used to compare indexes within group 2 birds (control and atropine and propranolol simultaneous treatment), and (iii) Mann-Whitney-Wilcoxon tests were used to compare indexes between birds of group 1 and 2. Furthermore, we tested whether baseline HR differed between birds of group 1 and 2 using a Mann-Whitney test comparing the mean HR measured 50 min after saline injection in birds from group 1 with the HR of control birds from group 2. No difference was detected between the two groups of individuals ($W = 5$, $P = 1.00$). The significance threshold was 0.05 for all tests.

Results

Frequency Bands and Respiration Rate

All individuals showed the expected LF and HF peaks (fig. 1), though the exact frequencies of the peaks varied slightly among birds: LF peaks appeared between 0.05 and 0.12 Hz, and HF peaks occurred between 0.43 and 0.85 Hz. LF and HF ranges were therefore delimited as 0.02–0.15 and 0.15–1.5 Hz, respectively. Resting respiration rate measured in five birds averaged 50 ± 5 breaths min^{-1}, ranging 44–55 breaths min^{-1}, which corresponds to frequencies of 0.73–0.92 Hz. Respiration occurred within the same frequency range as the observed HF peaks.

ANS Balance during Rest

ANS balance (combined PNS and SNS activity) during rest was quantified by comparing HR and HRV indexes in resting con-

Figure 1. *A*, Power spectra of heart rate variability in a wild-caught streaked shearwater. *Top*, resting controls (no injection); *bottom*, after injection of a parasympathetic nervous system (PNS) blocker (atropine) and a sympathetic nervous system (SNS) blocker (propranolol). *B*, Power spectra of heart rate variability of a wild-caught streaked shearwater. *Top*, after injection of a saline solution; *middle*, after injection of a PNS blocker (atropine); *bottom*, after injection with an SNS blocker (propranolol). LF = low frequency; HF = high frequency; ANS = autonomic nervous system.

trols with those following total ANS blockade (no PNS or SNS activity). ANS balance during rest was dominated by the PNS, as HR in resting controls was 53% lower than during total ANS blockade ($t = -14.01$, df = 4, $P = 0.0002$; table 2; fig. 2), and CV was fivefold higher in resting controls than during total ANS blockade ($t = 3.323$, df = 4, $P = 0.029$; table 2), indicating the presence of substantial ANS-mediated HRV.

In addition, HRV, as measured by LF (which reflects combined PNS and SNS activity), HF (which reflects PNS activity), and total power (which reflects predominately PNS activity), was significantly higher in resting controls than during ANS blockade (LF: $t = 4.129$, df = 4, $P = 0.015$; HF: $t = 3.569$, df = 4, $P = 0.023$; total: $t = 4.075$, df = 4, $P = 0.015$; table 3; figs. 3, 4), indicating strong ANS activity in HRV during rest, with a particularly strong effect of the PNS. Furthermore, the LF:HF ratio was marginally lower during rest, also pointing to PNS-dominated ANS balance ($t = -2.251$, df = 4, $P = 0.088$; table 3; fig. 5).

SDNN (reflecting combined PNS and SNS activity) and RMSSD (reflecting PNS activity) indexes of HRV were also higher in resting controls than during total ANS blockade (SDNN: $t = 6.71$, df = 4, $P = 0.003$; RMSSD: $t = 6169$, df = 4, $P = 0.004$; table 4), indicating strong ANS activity in HRV, though the SDNN:RMSSD ratio (which reflects the SNS-PNS balance) did not differ between resting controls and total ANS blockade ($t = 1.09$, df = 4, $P = 0.337$; table 4).

ANS Balance following Saline (Sham) Injection

ANS balance following injection was quantified by comparing HR and HRV indexes following saline (sham) injection with those following total ANS blockade (no PNS or SNS activity). ANS balance following saline injection was also dominated by PNS activity, as HR after saline injection was 28% lower than during total ANS blockade ($W = 0$, $P = 0.016$; table 2; fig. 2). In addition, CV was significantly higher after saline injection than during total ANS blockade ($W = 19$, $P = 0.032$; table 2), indicating the presence of substantial ANS-mediated HRV following saline injection.

Frequency domain analyses also revealed significant ANS activity in HRV following saline injection, as LF (which reflects combined PNS and SNS activity), HF (which reflects PNS activity), and total power (which predominately reflects PNS activity) were significantly higher than during total ANS blockade ($W = 20$ for all, $P = 0.019$, 0.020, and 0.016, respectively; table 3; figs. 3, 4). LF:HF ratio was lower following saline injection compared to total ANS blockade, indicating that the PNS dominated cardiac control even after saline injection ($W = 1$, $P = 0.032$; table 3; fig. 5).

Time domain analyses revealed substantial ANS activity in HRV following saline injection reflected in higher SDNN (which reflects combined PNS and SNS activity) and RMSSD (which reflects PNS activity) values compared to those when total ANS was blocked ($W = 20$ for both, $P = 0.015$ and 0.011, respectively; table 4), though the SDNN:RMSSD ratio (which reflects combined PNS and SNS activity) did not differ between the two treatments ($W = 14$, $P = 0.413$; table 4).

Effect of an Acute Stressor on ANS Balance

The effect of the stress of injection was quantified by comparing HR and HRV indexes following saline injection (combined PNS or SNS activity during acute stress) with those in resting controls (combined PNS and SNS activity at rest). After the

Table 1: List of abbreviations and indexes

Abbreviation	Definition
HR	Heart rate
HRV	Heart rate variability
ECG	Electrocardiogram
ANS	Autonomic nervous system
PNS	Parasympathetic nervous system
SNS	Sympathetic nervous system
IBI	Inter(heart)beat interval
CV	Coefficient of variation of IBI
LF power	A frequency domain index of HRV representing spectral power of low-frequency oscillations (reflects PNS and SNS activity)
HF power	A frequency domain index of HRV representing spectral power of high-frequency oscillations (reflects PNS activity)
LF:HF ratio	The ratio between LF and HF power (reflects SNS-PNS balance)
SDNN	A time domain index of HRV representing the standard deviation of all IBIs (reflects PNS and SNS activity)
RMSSD	A time domain index of HRV representing the standard deviation of the differences between successive IBIs (reflects PNS activity)
SDNN:RMSSD ratio	A time domain index of HRV representing the ratio between SDNN and RMSSD indexes (reflects SNS-PNS balance)

Table 2: HR and CV of HR in streaked shearwaters

	Resting	Control (saline)	SNS blockade (propranolol)	PNS blockade (atropine)	SNS + PNS blockade (atropine + propranolol)
HR (bpm)	134 ± 4[a,b]	205 ± 38[a]	181 ± 36[a]	324 ± 31	286 ± 27[b]
CV (%)	15 ± 6[a]	9 ± 3[a]	12 ± 4[a]	6 ± 2[a]	3 ± 3[b]

Note. Parameters measured resting conditions after the injection of a saline solution or of autonomic blockers ($n = 5$ birds in each group except for saline, in which $n = 4$). The values are presented as mean ± SD. See table 1 for definitions of abbreviations.

[a] Significantly different from total ANS (SNS + PNS) blockade ($P < 0.05$).

[b] Significantly different from the saline control ($P < 0.05$).

saline injection, there was a substantial shift in ANS balance with a reduction in PNS and/or increase in SNS activity, compared with control birds. First, HR was 53% higher following saline injection than in the resting controls ($W = 0$, $P = 0.0159$; table 2; fig. 2), indicating a reduction in relative PNS activity. CV (which reflects combined PNS and SNS activity) was 40% lower compared to the resting state, though this effect was not significant ($W = 11$, $P = 0.905$; table 2). But, total spectral power (containing a predominant PNS contribution) significantly decreased by 81%, LF power (which reflects combined PNS and SNS activity) decreased by 82%, and HF power (which reflects PNS activity) decreased by 81%, compared to the resting controls ($W = 20$ for LF and 19 for HF and total power, $P = 0.016$, 0.037, and 0.032, respectively; table 3; figs. 3, 4), suggesting PNS inhibition, though the LF : HF ratio, which reflects ANS balance, did not change ($W = 13$, $P = 0.556$; table 3; fig. 5).

In addition, RMSSD, which reflects PNS activity, decreased by 69%, and SDNN, which reflects both PNS and SNS activity, decreased by 65% compared to the control ($W = 20$ for both, $P = 0.016$ and 0.095, respectively; table 4), suggesting PNS inhibition, though the SDNN : RMSSD ratio was similar under both conditions ($W = 0$, $P = 0.730$; table 4).

SNS Drive

SNS drive was quantified by comparing HR and HRV indexes following PNS blockade with those following total ANS blockade. HR was elevated by 13% after PNS blockade (leaving only SNS activity) compared to during total ANS blockade, indicating the presence of some SNS activity, though the difference was not significant ($W = 21$, $P = 0.095$; table 2; fig. 2). CV, however, was significantly higher during PNS blockade than during total ANS blockade ($W = 23$, $P = 0.032$; table 2), indicating the presence of some SNS-mediated HRV.

Frequency domain analyses revealed some SNS drive in the LF band, as LF power was marginally higher during PNS blockade compared to during total ANS blockade ($W = 22$, $P = 0.059$; table 3; fig. 3). Total spectral power was also marginally higher during PNS blockade than during total ANS blockade ($W = 21$, $P = 0.095$; table 3), pointing to some contribution of the SNS to HRV. As expected, HF power, which reflects only PNS activity, was similar during PNS blockade and total ANS blockade ($W = 10$, $P = 0.671$; table 3; fig. 4). The LF : HF ratio, however, was significantly higher during PNS blockade than during total ANS blockade ($W = 23$, $P = 0.032$; table 3; fig. 5) because some LF power (which in this case reflected purely SNS activity) remained, whereas HF power (which reflects only PNS activity) was almost eliminated.

Time domain indexes, however, did not detect the presence of any SNS drive, as the SDNN during PNS blockade (which in this reflected purely SNS activity) was not higher than during total ANS blockade ($W = 18$, $P = 0.232$; table 4) nor was the SDNN : RMSSD ratio (which reflects the SNS-PNS balance) higher during PNS blockade than during total ANS blockade ($W = 19$, $P = 0.222$; table 4). RMSSD, which reflects only PNS tone, was also not different when the PNS was blocked than during total ANS blockade ($W = 12.5$, $P = 1$; table 4).

PNS Drive

PNS drive was quantified by comparing HR and HRV indexes following SNS blockade with those following total ANS

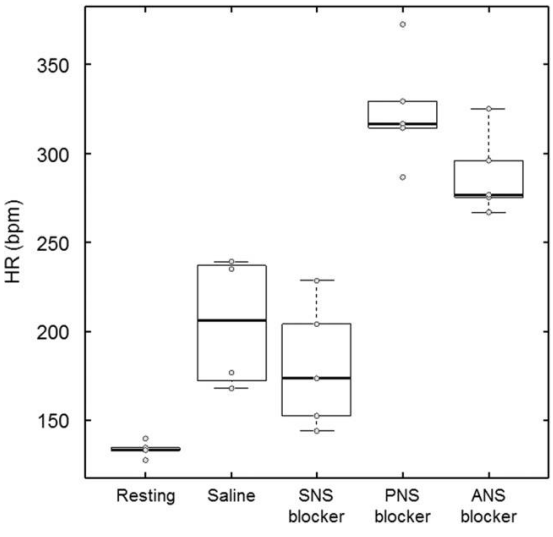


Figure 2. Boxplots of heart rate (HR; beats per minute [bpm]) in wild-caught streaked shearwaters. HR measured under resting conditions ($n = 5$) and after injection of a saline solution ($n = 4$), a sympathetic nervous system (SNS) blocker (propranolol), a parasympathetic nervous system (PNS) blocker (atropine), and both blockers (total autonomic nervous system [ANS] blockade, atropine + propranolol; $n = 5$). A color version of this figure is available online.

Table 3: Frequency domain indexes of heart rate variability in streaked shearwaters

	Resting	Control (saline)	SNS blockade (propranolol)	PNS blockade (atropine)	SNS + PNS blockade (atropine + propranolol)
Total power (ms^2)	1,213 ± 658[a,b]	231 ± 215[a]	557 ± 390[a]	33 ± 43	8 ± 9[b]
LF (ms^2)	877 ± 470[a,b]	161 ± 154[a]	291 ± 194[a]	21 ± 26	5 ± 5[b]
HF (ms^2)	213 ± 133[a,b]	40 ± 38[a]	225 ± 242[a]	.4 ± .6	.4 ± .1[b]
LF:HF	4.5 ± 2.1	3.2 ± 2.4[a]	2.6 ± 2.5[a]	49.5 ± 48.8[a]	12.6 ± 6.9[b]

Note. Total, low-frequency (LF), and high-frequency (HF) powers and the LF:HF ratio of streaked shearwaters under resting conditions after the injection of a saline solution or of autonomic blockers ($n = 5$ birds in each group except for saline, in which $n = 4$). The values are presented as mean ± SD. See table 1 for definitions of abbreviations.

[a] Significantly different from total ANS (SNS + PNS) blockade ($P < 0.05$).

[b] Significantly different from the saline control ($P < 0.05$).

blockade. PNS drive was more pronounced than SNS drive, as HR was 37% lower following injection with an SNS blocker (leaving only PNS activity) than during total ANS blockade ($W = 0$, $P = 0.008$; table 2; fig. 1), and HR during PNS blockade did not differ from that during total ANS blockade (table 2). Furthermore, CV was also substantially higher during SNS blockade than during total ANS blockade ($W = 25$, $P = 0.008$; table 2).

Frequency domain analyses also revealed pronounced PNS drive, with significantly higher LF power, HF power, and total spectral power present during SNS blockade than during total ANS blockade ($W = 25$ for both, $P = 0.008$ for HF and 0.012 for LF and total power; table 3; figs. 3, 4). The LF:HF ratio was lower during SNS blockade than during total ANS blockade ($W = 1$, $P = 0.016$; table 3; fig. 5), indicating the disproportionate influence the PNS had on increasing HF spectral power.

Time domain analyses also provided evidence for strong PNS drive in both SDNN and RMSSD indexes, as both values were significantly higher during SNS blockade than during total ANS blockade ($W = 25$ for both, $P = 0.010$ and 0.008, respectively; table 4). The SDNN:RMSSD ratio was similar during SNS blockade and during total ANS blockade ($W = 16$, $P = 0.548$; table 4), reflecting the fact that SNS drive was negligible in any time domain indexes.

Reciprocal Inhibition of the PNS and SNS

Reciprocal inhibition of the ANS branches was quantified by comparing HRV indexes following single-branch blockade (isolated PNS or SNS activity) with those following saline injection (combined PNS and SNS activity). SNS blockade did not significantly elevate LF, HF, and total spectral power compared to the saline treatment ($V = 1$ for LF and 0 for HF and total powers, $P = 0.250$ for LF and 0.125 for HF and total powers; table 3; figs. 3, 4), and the LF:HF ratio also remained the same ($V = 4$, $P = 0.875$; table 3; fig. 5). Similarly, though SDNN and RMSSD values were slightly higher during SNS blockade than following saline injection, the effect was not significant ($V = 0$ for both, $P = 0.125$ for both; table 4). SDNN:RMSSD values were also similar between SNS-blocked and saline-injected treatments ($V = 7$, $P = 0.625$; table 4).

PNS blockade resulted in a slight nonsignificant decrease in LF, HF, and total spectral power compared to the saline treatment ($V = 10$, $P = 0.125$ for all; table 3; figs. 3, 4). Similarly, after PNS blockade, SDNN and RMSSD values were slightly depressed following saline injection, although not significantly so ($V = 10$, $P = 0.125$ for both,; table 4). SDNN:RMSSD values were also similar between groups ($V = 2$, $P = 0.375$; table 4).

Discussion

To the best of our knowledge, this study is the first to investigate the influence of the ANS on the cardiac function of a wild seabird. Furthermore, this is one of the first avian studies

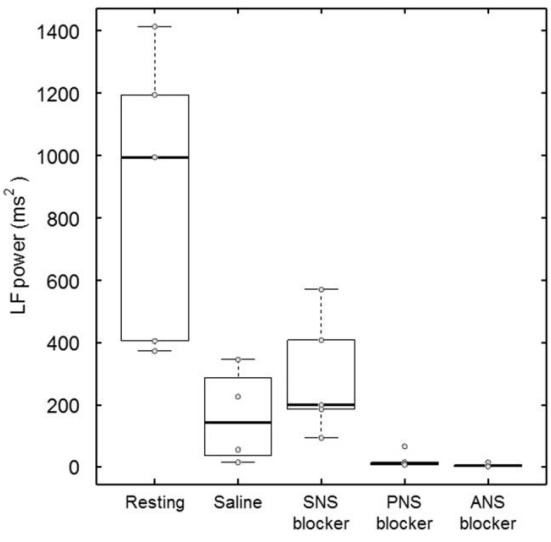


Figure 3. Boxplots of spectral power of low-frequency (LF) oscillations (0.02–0.15 Hz) in interheartbeat intervals in wild-caught streaked shearwaters. LF power measured under resting conditions ($n = 5$) and after injection of a saline solution ($n = 4$), a sympathetic nervous system (SNS) blocker (propranolol), a parasympathetic nervous system (PNS) blocker (atropine), and both blockers (total autonomic nervous system [ANS] blockade, atropine + propranolol; $n = 5$). A color version of this figure is available online.

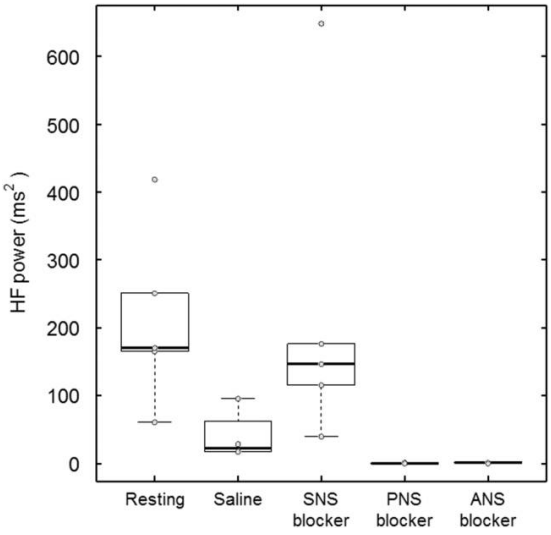


Figure 4. Boxplots of spectral power of high-frequency (HF) oscillations (0.15–1.5 Hz) in interheartbeat intervals in wild-caught streaked shearwaters. HF power measured under resting conditions ($n = 5$) and after injection of a saline solution ($n = 4$), a sympathetic nervous system (SNS) blocker (propranolol), a parasympathetic nervous system (PNS) blocker (atropine), and both blockers (total autonomic nervous system [ANS] blockade, atropine + propranolol; $n = 5$). A color version of this figure is available online.

to experimentally isolate the contributions of PNS and SNS drive by comparing cardiac parameters during single-branch blockade to those occurring under total ANS blockade. The time and frequency domain analyses of HR and HRV from our experiment indicate that in the streaked shearwater, (i) the ANS's influence on HR and HRV following capture is dominated by the PNS rather than the SNS, (ii) one branch of the ANS does not strongly suppress the actions of the other, and (iii) following an acute stressor (the injection of saline), PNS activity is reduced but still contributes strongly to HR and HRV indexes, whereas SNS activity does not. These findings notably contrast with results from Dickens and Romero (2009), who detected an important stress-induced SNS drive on HR and HRV of wild birds recently introduced into captivity. The streaked shearwaters studied here were also recently introduced into captivity but displayed clear PNS dominance both at rest and after an acute stressor, further underscoring the strength of the PNS drive on HR and HRV in this species and also indicating that the birds were not critically stressed.

PNS and SNS Activity in HR

Our analyses demonstrate that the PNS's effect in slowing HR in wild-caught streaked shearwaters was stronger than the SNS's effect in accelerating HR, both during rest and following saline injection, as HR was lower during rest and following saline injection than it was during total ANS blockade. These findings are consistent with several previous studies that have found PNS control to predominate in HR of undomesticated birds (e.g., Cyr et al. 2009; Yamamoto et al. 2009; Shah et al. 2010; reviewed in Taylor et al. 2014). On the contrary, domesticated chickens, which were long selectively bred under artificial conditions, appear to have a dominant SNS influence on HR (Matsui and Sugano 1987).

PNS and SNS Drive in HRV

As expected, we found that the ANS is the cause of most of the HRV measured in the streaked shearwaters, as total ANS blockade dramatically reduced HF, LF, and also total power of HRV. But which branch of the ANS contributed most to HRV? We found that PNS drive, by far, dominated HRV from almost all parameters examined in this study. When only the PNS was active, HF, LF, and total power were high and much higher than during total ANS blockade, indicating that PNS activity contributes most of the HRV at both the LF range and the HF range. Furthermore, when the PNS was blocked, HF power was reduced to the level exhibited during total ANS blockade, indicating that, as expected, the PNS is the sole contributor to HF oscillations. Also, LF and total power were dramatically reduced during PNS blockade (though not to the level observed during total ANS blockade), again indicating important PNS contributions. As expected, we also found significant PNS con-

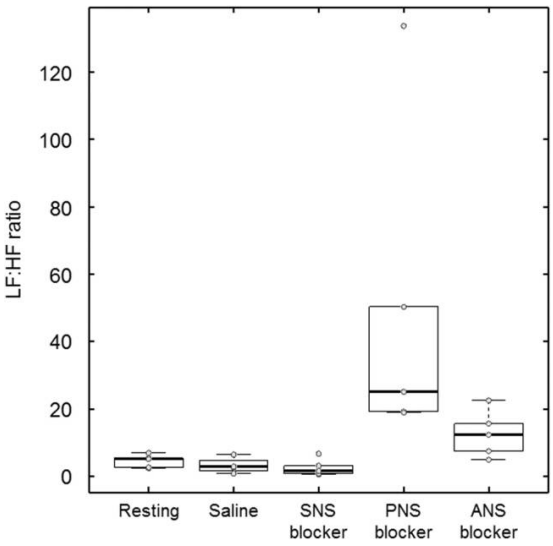


Figure 5. Boxplots of the ratio in spectral power of low-frequency (LF; 0.02–0.15 Hz) and high-frequency (HF; 0.15–1.5 Hz) oscillations in interheartbeat intervals in wild-caught streaked shearwaters. LF : HF ratio measured under resting conditions ($n = 5$) and after injection of a saline solution ($n = 4$), a sympathetic nervous system (SNS) blocker (propranolol), a parasympathetic nervous system (PNS) blocker (atropine), and both blockers (total autonomic nervous system [ANS] blockade, atropine + propranolol; $n = 5$). A color version of this figure is available online.

Table 4: Time domain indexes of heart rate variability in streaked shearwaters

	Resting	Control (saline)	SNS blockade (propranolol)	PNS blockade (atropine)	SNS + PNS blockade (atropine + propranolol)
SDNN (ms)	31 ± 10[a,b]	12 ± 5[a]	29 ± 24[a]	3 ± 1	2 ± .5[b]
RMSSD (ms)	29 ± 10[a]	10 ± 4[a]	33 ± 33[a]	2 ± .7	2 ± .2[b]
SDNN : RMSSD	1.2 ± .2	1.2 ± .2	1.1 ± .3	1.5 ± .6	1.0 ± .2

Note. SDNN, RMSSD, and the SDNN : RMSSD ratio of streaked shearwaters under resting conditions after the injection of a saline solution or of autonomic blockers ($n = 5$ birds in each group except for saline, in which $n = 4$). The values are presented as mean ± SD. See table 1 for definitions of abbreviations.

[a] Significantly different from total ANS (SNS + PNS) blockade ($P < 0.05$).

[b] Significantly different from the saline control ($P < 0.05$).

tribution in the RMSSD index, which reflects PNS activity. Finally, the SDNN index, which reflects activity of both PNS and SNS, was also higher when only the PNS was active than during total ANS blockade, indicating predominate PNS activity.

SNS drive in HRV was also present but to a substantially lesser extent than PNS drive. The SNS contributed significantly to CV. Furthermore, the LF : HF ratio was significantly lower during SNS blockade than during total ANS blockade (indicating disproportionate reduction in LF power), while it was significantly higher during PNS blockade (indicating disproportionate reduction in HF power) than during total ANS blockade. This pattern confirms the assumption that the SNS contributes to LF power in streaked shearwaters, though the effects were not large enough to be detected in LF power alone. Furthermore, SNS activity was not detected in RMSSD and SDNN indexes. Shah et al. (2010) found similar results in emu hatchlings, as PNS blockade produced a significant decline in LF and HF spectral power of HRV, which did not change with the addition of SNS blockade.

Reciprocal Suppression of PNS and SNS in HRV

Reciprocal suppression of PNS and SNS can be revealed when pharmacologically blocking one branch of the ANS results in higher activity of the other branch of the ANS (reflected in HRV indexes) compared to when both the PNS and SNS are active (e.g., following saline injection). When we compared single-branch blockade with the saline sham treatment, however, we found no evidence for reciprocal suppression of the PNS and SNS contribution to HRV. Evidence for SNS inhibition of the PNS effects on HRV has previously been shown for emu *Dromaius novaehollandiae* hatchlings (Shah et al. 2010), great cormorants (Yamamoto et al. 2009), and horses *Equus ferus caballus* (Kuwahara et al. 1996), in which spectral power increased after animals received an SNS blocker. In our study this effect was not significant, suggesting that this may not be the rule for all PNS-dominant birds and probably mammals too.

ANS Balance in HRV during Resting Conditions in Captivity

Shearwaters exhibited strongly dominating PNS activity while at rest, as indicated by an HR that was much lower at rest than during total ANS blockade. In addition, several indexes of PNS-influenced HRV were significantly higher during rest than during total ANS blockade, including CV, LF, HF, and total power, as well as SDNN and RMSSD. Though we found strongly dominant PNS activity, measurements in our study reflect ANS activity following several days of sustained exposure to the stress of captivity in a novel environment. A previous study of ANS activity in captured wild starlings (*Sturnus vulgaris*) showed that during the first few days of captivity, the birds had higher HR and reduced HRV compared to birds that were acclimated to captivity, indicating a shift in ANS balance toward SNS dominance (Dickens and Romero 2009). Furthermore, starlings exposed to chronic stress for more than 2 wk had elevated basal HR (though no effect on HRV), indicating an increase in SNS activity (Cyr et al. 2009). Here the shearwaters did not exhibit SNS dominance after a few days of captivity, so they either (1) recovered from the stress of initial capture and quickly acclimated to captivity or (2) have a much stronger PNS dominance than the starlings (Dickens and Romero 2009). Both cases indicate PNS dominance in shearwaters, though the latter scenario would suggest that unstressed shearwaters in a natural setting would have even more pronounced PNS dominance in HR and HRV control.

ANS Balance in HRV following an Acute Stressor

As expected, we found an ANS-mediated stress response following injection, as HRV changed in a manner consistent with dramatic PNS withdrawal: total spectral power, LF power, and HF power decreased dramatically compared to resting controls. In addition, RMSSD (which reflects PNS activity) and SDNN (which reflects both PNS and SNS activity) also decreased substantially following injection compared to resting controls. HR increased following saline injection compared to resting controls, which could reflect PNS withdrawal but also SNS activation. Had the SNS been strongly activated, however, we would have seen an increase (or at least less of a decrease) in the spectral power of oscillations in the LF range. Instead we found decreases in HRV of a similar magnitude across all frequencies and indexes that reflect either solely PNS activity or at least predominantly PNS activity. Thus, the relatively rapid stress response observed in the 9-min interval following saline injection appeared to include primarily a severe reduction in PNS drive.

Exposure to an acute stressor has been hypothesized to activate a hierarchical response strategy, first causing the release of a vagal brake, which immediately reduces PNS drive, increasing HR and decreasing HRV (polyvagal theory; Porges 1992, 2007). Porges (1992, 2007) suggests that this PNS brake comprises relatively recently evolved myelinated vagal nerve fibers extending from the nucleus ambiguous (NA) to the heart, forming the ventral vagal complex, whereas the more ancestral unmyelinated vagus that forms the dorsal vagal complex (DVN) regulates the freeze response.

Though Porges (1992) suggested that the myelinated vagal brake coming from the NA exists only in mammals, evidence for the existence of the myelinated vagal brake in birds and other nonmammals is relatively strong given the substantial afferent and efferent myelinated vagal nerve fibers found in domesticated fowl (Abdalla and King 1979) and other nonmammals (Taylor et al. 1999, 2001). Furthermore, the respiratory arrhythmia in HRV is attributed to the fast-conducting myelinated vagal nerves (Stauss 2003; Taylor et al. 2014), and we found a strong respiratory arrhythmia signal in shearwaters, as have other studies on birds (Yamamoto et al. 2009; Shah et al. 2010) and several other nonmammalian species including lizards and snakes (Taylor et al. 1999, 2001, 2014; Campbell et al. 2006). In addition, respiratory arrhythmia in HRV appears to depend on cardiac vagal preganglionic neurons (CVPNs) being concentrated not only within the DVN but also outside of it, with each group exerting distinct actions on the heart. Neuroanatomical studies of a duck (Blogg et al. 1998) and several other nonmammalian taxa (Taylor et al. 1992, 1999, 2001; Campbell et al. 2006) have indeed found CVPN clusters both within and outside of the DVN and measured separate effects on the heart (Taylor et al. 1992). This further points to the existence of a vagal brake in diverse nonmammalian taxa (further discussed in Campbell et al. 2006; Taylor et al. 2014).

The stress response of injection on ANS activity has received little attention in birds so far. Our study is one of the first to present evidence for an avian vagal brake stress response. Cyr et al. (2009) found that intramuscular saline injections in wild-caught starlings produced no stress-induced increase in HR integrated across the 15 min that followed injection, though short-term HR elevations were observed that lasted ca. 3–5 min (effects of injection on HRV were not reported). This may be similar to what we detected in shearwaters in our study. Moreover, when Cyr et al. (2009) restrained the starlings in an opaque cloth bag for 15 min, HR increased and HRV decreased compared to unrestrained birds, indicating a shift away from PNS dominance, though it is unclear whether this shift is due to increased SNS drive or PNS withdrawal (i.e., release of the vagal brake), as observed in our study. However, Cyr et al. (2009) showed that when starlings were chronically stressed over a 16-d period and then exposed to an acute stressor, the resulting HR increase was caused by PNS withdrawal, not SNS activation. This is similar to what we observed in our birds, which were also potentially chronically stressed (newly in captivity) and then exposed to an acute stressor (injection). It could be that ANS responses to acute stressors in chronically stressed birds employ primarily a vagal brake (i.e., PNS withdrawal) mechanism, whereas birds that are not chronically stressed employ both PNS withdrawal and SNS activation. Indeed, both Cyr et al. (2009) and Dickens and Romero (2009) demonstrated that chronically stressed birds exhibit an attenuated HR elevation in response to an acute stressor.

ANS Balance in Relation to Ecology and Life History

We found that the PNS had a much stronger effect than the SNS on cardiac parameters in recently captured streaked shearwaters. The resting HR measured in this study was 134 bpm, which is substantially lower than the range of 176–182 bpm predicted by the allometric equation proposed by Lasiewski and Calder (1971), and may actually be even lower in relaxed birds in their natural habitat. Their low HR may be due to a disproportionately large heart that efficiently distributes blood throughout their body, as has also been suggested for great cormorants (Yamamoto et al. 2009). Indeed, Y. Niizuma (unpublished data) measured a heart mass in streaked shearwaters that accounts for 1.2% ± 0.4% of the birds' body mass, which is high for seabirds of that size (0.9%–1% of body mass; Bishop 1997). Streaked shearwaters fly long distances during both the breeding season (up to 1,000 km; Yoda et al. 2014) and migration (to up to 5,200 km; Yamamoto et al. 2010). During periods of insufficient wind, they may flap intensely, experiencing tachycardia for long bouts, as observed in Cape gannets (Ropert-Coudert et al. 2006), and thus requiring a high-performing cardiovascular system. In birds and mammals, a low HR at rest has been associated with a large rate of HR increase during exercise or stressful conditions (Bevan et al. 1997; Machida and Aohagi 2001). Such flexibility in HR is often found in species that at rest display PNS dominance in cardiac function (Kuwahara et al. 1996; Yamamoto et al. 2009), consistent with what we observed in the streaked shearwater.

Furthermore, streaked shearwaters begin breeding late (ca. age 4), produce, at most, one offspring per year, and so should prioritize their survival over any individual reproductive attempt so they can live long enough to maximize their lifetime reproductive success (Medawar 1952; Goodman 1974). To achieve this, long-lived species such as seabirds employ physiological strategies that minimize the "wear and tear" effect of cumulative cellular damage by prioritizing self-maintenance processes that help mitigate somatic deterioration (discussed in Apanius et al. 2008). Maintaining high PNS tone is consistent with such a strategy (Epel et al. 2006; Zhang and Zhang 2009; Révész et al. 2014). The association between several stress-related physiological parameters potentially involved in aging is being increasingly studied in model and nonmodel animals (e.g., Costantini et al. 2011; Ballen et al. 2012; Beery et al. 2012; Geiger et al. 2012; Haussmann et al. 2011; Hau et al. 2015; reviewed in Monaghan 2014), including seabirds (e.g., Lecomte 2010; Elliot et al. 2014), but thus far the specific

role of ANS balance has rarely been addressed. Future studies will reveal whether relative PNS dominance correlates with life span across species.

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APPENDIX

Supplementary Material

Dose Determination Study

In September, four birds were injected with increasing consecutive doses of atropine (0.05, 0.1, 0.3, 0.5 mg kg^{-1}) and propranolol (0.1, 0.2, 0.3, 0.4 mg kg^{-1}) at an interval of 24 h between the two treatments. Doses were decided according to a study on chickens (Matsui and Sugano 1987). The electrocardiogram (ECG) was monitored with an externally attached ECG data logger (UME-ECG, 12-bit resolution, 55 mm × 12 mm, 14.75 g, Little Leonardo) at a sampling interval of 7.8 ms. Doses that exerted the desired inhibitory effects on the autonomic nervous system (ANS) and that permitted rapid stabilization and recovery of heart rate (HR) after injection were chosen. We chose for the experiment to administer saline (0.9%) and atropine (0.3 mg kg^{-1}) injections via a liquid volume of 0.3 mL. As we found the effect of propranolol on this species to be severe, the 0.2 mg kg^{-1} dose was delivered in a larger volume of 0.5 mL.

Selection of Intervals for ECG Analysis

Saline injection significantly elevated HR above the resting level during phases 1 and 2 (Mann-Whitney tests, $W = 0$ and 1, $P = 0.016$ and 0.032, respectively) and reduced coefficient of variation (CV) during phase 1 although not significantly so ($W = 11$, $P = 0.905$). But the effect waned with time, with HR reaching the resting level after approximately 40 min after the injection (phase 3, $W = 5$, $P = 1$). The maximum effects of the autonomic blockers on HR and CV were reached during phase 1. Atropine (parasympathetic nervous system blocker) significantly elevated HR and reduced CV during phase 1, compared to saline controls (Wilcoxon signed-rank test, $V = 0$ and 40, $P = 0.0039$ and 0.0391, respectively). Conversely, propranolol (sympathetic nervous system blocker) had no significant effect on HR or CV compared to saline controls even during phase 1 when its effect was at maximum ($V = 43$ and 32, $P = 0.131$ and 0.695, respectively). The effect of total ANS blockade (the simultaneous injection of atropine and propranolol) was very similar to that of atropine alone: HR was significantly elevated during phase 1 and also for phase 2 (Mann-Whitney tests, $W = 2$ and 0, $P < 0.001$ and 0.029, respectively), and CV was significantly reduced for phase 1 and also for phase 2 ($W = 113$ and 16, $P < 0.001$ and 0.029, respectively). In summary, the period during which autonomic blockers were having their maximum effect was phase 1, so frequency and time domain indexes were calculated for phase 1 for all subsequent analyses in injected birds and for the rest phase in resting birds.

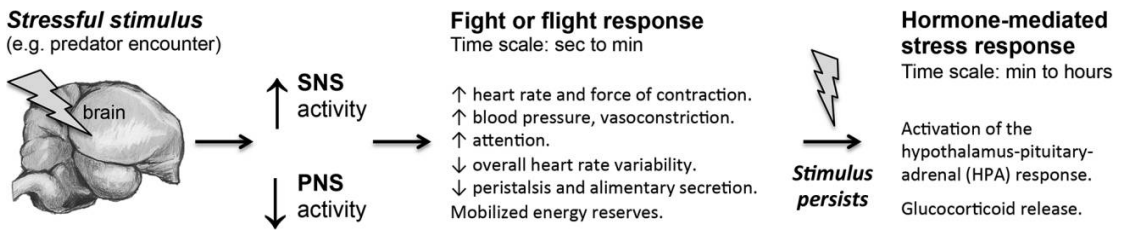

Figure A1. Summary of the main physiological effects of the autonomic nervous system–mediated stress response, summarized from Porges (1992) and Wingfield (2013). SNS = sympathetic nervous system; PNS = parasympathetic nervous system.

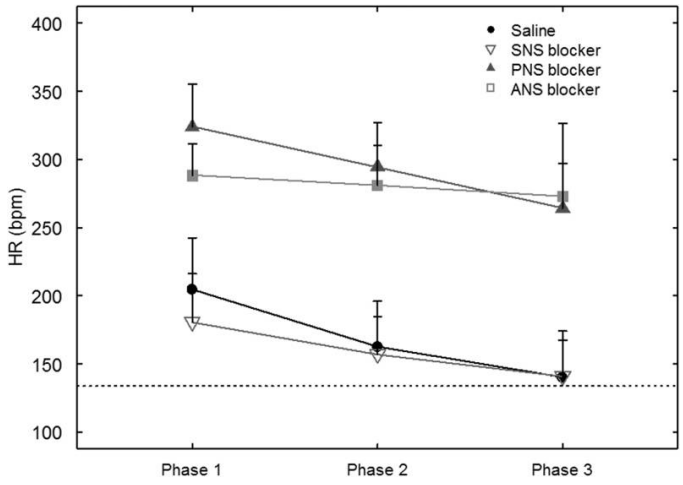

Figure A2. Heart rate (HR; beats per minute [bpm]) over 1 h of electrocardiogram (ECG) recordings after injection of a saline solution ($n = 4$) and of a sympathetic nervous system (SNS) blocker (propranolol), a parasympathetic nervous system (PNS) blocker (atropine), and total autonomic nervous system (ANS) blockade (atropine + propranolol; $n = 5$) in wild-caught streaked shearwaters. Points correspond to the 9-min mean HR calculated for phases 1, 2, and 3, corresponding to 4–15, 16–31, and 35–49 min after injection, respectively. The dotted line corresponds to resting HR (mean value 30–50 min after ECG logger attachment; $n = 5$). A color version of this figure is available online.

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